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Cigarette smoke condensate induces MMP-12 gene expression in airway-like epithelia

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Abstract

Cigarette smoke (CS)-induced emphysema is attributable to matrix metalloproteinase-12 (MMP-12) in mice, however, a relationship between CS and MMP-12 is absent in human emphysema. Here, we show that cigarette smoke condensate (CSC) induces MMP-12 gene expression in airway-like epithelia through a hydrogen peroxide (H_2O_2)-dependent pathway involving NADPH oxidase, AP-1, and TNF-α. Cigarette smoke condensate-induced H_2O_2 production and MMP-12 gene expression were inhibited by apocynin, a specific inhibitor of NADPH oxidases, while 3-aminobenzamide, an inhibitor of AP-1, attenuated CSC-induced MMP-12 gene expression. Messenger RNAs encoding phagocytic NADPH oxidase components and a homologue of p67*phox*, p51 (*NOXA1*), were detected, while mRNA of dual oxidase (Duox)1 was unchanged by CSC. Enbrel, an inhibitor of TNF-α function, reduced CSC-induced H_2O_2 production and MMP-12 expression. These findings provide novel evidence of a direct relationship between CS exposure and MMP-12 in human airway epithelia and suggest several targets for modulation of this potentially pathogenic pathway.
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Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death in the United States and is responsible for mortality of 16 million victims worldwide annually [1]. While the causes and pulmonary locations of this condition vary, the manifestations are commonly characterized by airflow obstruction that is not completely reversible [1]. The latter feature is especially attributable to emphysema, a form of COPD that is characterized by pulmonary parenchymal damage, and alveolar enlargement and coalescence. These structural changes reduce the radial traction extended from alveoli to airways, thereby limiting airflow during expiration. Chronic cigarette smoke (CS) exposure, which is associated with chronic inflammation and oxidative stress within lungs [2], is regarded as the primary cause of emphysema [1]. In an effort to prevent emphysema or halt its progression following cessation of cigarette smoking, research efforts have sought to identify the cells, inflammatory mediators, and oxidant-mediated events that are responsible for its development. Some of these studies have been guided by recognition that proteases, such as neutrophil elastase [3] and matrix metalloproteinase-12 (MMP-12) [4], may mediate the destructive effects of CS in lung.

Matrix metalloproteinase-12 or macrophage metalloelastase derives its name from its predominant expression in macrophages and its ability to degrade elastin [5]. This enzyme is activated when the N-terminal propeptide domain, which is physically associated with

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the catalytic domain through coordination to the Zn^{2+}-containing active site, dissociates and is cleaved from the remainder of the protein. Matrix metalloproteinase-12 has been implicated as an etiological factor in emphysema, because MMP-12-deficient mice are resistant to CS-induced alveolar enlargement and macrophage infiltration into the lung [4]. Recent work in our laboratory showed that gene expression and secretion of MMP-12 occur in primary airway-like normal human bronchial epithelial cell (NHBEC) cultures [6]. These findings revealed a new potential source of pulmonary MMP-12 in vivo, where its gene expression may be induced by soluble stimuli, as observed in vitro [6]. Intracellular signaling pathways that are manifest as MMP-12 expression may involve oxidant-dependent mediators, since activator protein (AP)-1, a hydrogen peroxide (H_2O_2)-sensitive transcription factor [7], may bind to the 5′ promoter region of human MMP-12 [8]. Indeed, CS contains an abundance of oxidants [9] and the anatomical position of bronchial epithelia, at the boundary between internal and external lung environments, is optimal for direct exposure to CS. Alternatively, as suggested by studies in endothelial cells [10], oxidant burden associated with CS-induced emphysema can also be secondarily derived from CS stimulation of an endogenous, oxidant-producing enzyme, NADPH oxidase.

The phagocyte NADPH oxidase is a highly inducible, oxidant-producing enzyme that performs an essential host defense function in neutrophils and macrophages [11], which comprise the emphysematous inflammatory infiltrate [12]. Activation of NADPH oxidase depends on translocation of essential cytosolic components, p47*phox* (phagocyte oxidase), p67*phox*, and Rac1 or Rac2, to the plasma and phagolysosomal membranes, where they interact with the essential membrane-embedded flavocytochrome component, which is composed of p22*phox* and gp91*phox* proteins [11]. The active phagocyte NADPH oxidase primarily produces superoxide anions ($O_2^{\bullet}$) by oxidizing NADPH and enabling transmembrane electron-transfer through the flavocytochrome to oxygen. Spontaneous or superoxide dismutase (SOD)-catalyzed dismutation of $O_2^{\bullet}$ can follow to form H_2O_2, which can be oxidized by myeloperoxidase and conjugated to either chloride or bromide ions to form microbicidal hypochlorous or hypobromous acids, respectively. Absence of NADPH oxidase function is manifest as chronic granulomatous disease, a life-threatening condition characterized by persistent granulomas associated with bacterial and fungal infections [13]. Interestingly, modified versions of the phagocyte NADPH oxidase core component, gp91*phox*, are expressed and functional within non-phagocytic cells [14]. For instance, Geiszt et al. [15] showed that dual oxidase (Duox) 1 [16–18], a gp91*phox* homologue [16,17], can produce H_2O_2 in NHBECs. Additionally, homologues of p47*phox* and p67*phox*, referred to as p41 (NADPH oxidase organizer 1; NOXO1) and p51 (NADPH oxidase activator 1; NOXA1), respectively, have recently been described [19–21]. These findings raise the possibility that heterogeneous combinations of classical and novel components could comprise functional NADPH oxidases in a variety of cell-types.

The results of the study described here show that cigarette smoke condensate (CSC) induces MMP-12 expression in airway-like NHBEC cultures through an H_2O_2-dependent pathway involving NADPH oxidase and AP-1. In support of these findings, we provide genetic evidence that an NADPH oxidase(s) is present in airway-like NHBEC cultures. Further analyses suggest that tumor necrosis factor (TNF)-α cooperates with CSC to elicit both H_2O_2 production and MMP-12 expression.

Materials and methods

Derivation of airway-like NHBEC cultures. Differentiation of primary NHBECs (passage 1) into cultures bearing airway-like characteristics, including enhanced mucin production and secretion, has been described [22] and previously performed in this laboratory [6]. Briefly, NHBECs cultured on fibrillar collagen/human fibronectin membranes were maintained at an air-liquid interface for fourteen days in serum-free NHBEC culture medium (50% BEBM, Cambrex, Walkersville, MD; 50% DMEM (high glucose), Invitrogen, Grand Island, NY) supplemented with BEGM (Cambrex): hydrocortisone (0.5 μg/ml), insulin (5 μg/ml), transferrin (10 μg/ml), epinephrine (0.5 μg/ml), triiodothyronine (6.5×10^{-3} μg/ml), gentamicin-1000 (1 μl/ml), bovine pituitary extract (4 μl/ml), and additional supplements, including human epidermal growth factor-(EGF) (0.5 ng/ml; R&D Systems, Minneapolis, MN), *all-trans* retinoic acid (5×10^{-8} M; Sigma, St. Louis, MO), bovine serum albumin (1.5 μg/ml; Sigma), and amphotericin B (0.5 μg/ml; Sigma) to induce differentiation. Subsequently, cultures were analyzed by fluorescence microscopy, reverse transcription-polymerase chain reaction (RT-PCR), or real-time polymerase chain reaction (Taqman) analyses to evaluate oxidant production, gene expression, and mRNA abundance, respectively (see below).

Detection of oxidant production in NHBEC cultures with dihydrorhodamine 123. A fluorescent indicator, dihydrorhodamine 123 (DHR; Molecular Probes, Eugene, OR), was used to detect oxidant production in airway-like NHBEC cultures. This indicator emits a fluorescent signal when oxidized by H_2O_2. Following removal of serum-free NHBEC culture medium, cells were rinsed two times with Hanks' balanced salt solution (HBSS; containing Ca^{2+} and Mg^{2+}) prior to preloading cells with DHR (10 μM) in HBSS for 30 min at 37 °C. Next, treatment cocktails consisting of vehicle (DMSO; 0.04%), CSC (0.4 μg/ml) [23], CSC plus catalase (1500 U/ml), CSC plus apocynin (30 μM), or CSC plus Enbrel (etanercept; 50 ng/ml) in serum-free NHBEC culture medium were applied to both basal and apical sides of each culture. After incubation at 37 °C for 60 min, cultures were analyzed by fluorescence microscopy to visualize fluorescent emissions emanating from oxidized DHR.

Real-time polymerase chain reaction analyses. At time zero, serum-free medium bathing differentiated NHBEC cell cultures was aspirated and replaced with media containing a variety of compounds (see below). At designated time periods (2, 6, 24, and 48 h) following challenge with different media preparations, which included serum-free NHBEC culture medium containing vehicle (DMSO; 0.04%), CSC (0.4 μg/ml), CSC plus catalase (1500 U/ml), CSC plus apocynin (30 μM), CSC plus 3-aminobenzamide (1 mM), or CSC plus Enbrel

(etanercept; 50 ng/ml), conditioned-media samples were harvested and stored at −80 °C, and NHBECs were removed from membranes by adding trypsin containing 0.3% protease (Sigma) to both the basal (2.5 ml) and apical (1 ml) sides of cell layers. Cell pellets were washed once with 1× phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS), and processed for total RNA extraction using a RNeasy Qiagen mini kit (Qiagen; Valencia, CA). Total RNA pools were incubated with RNase-Free DNase (Qiagen) prior to determining total RNA concentrations by spectrophotometry. The concentration of total RNA in each sample was adjusted to 50 ng/μl in preparation for gene expression analysis by real-time polymerase chain reaction (Taqman).

Total RNA (125 ng) was added to standard Taqman reagents (Applied Biosystems; Branchburg, NJ) and the appropriate probe and primers (Table 1) to measure mRNA species of human MMP-12, Duox1, and TNF-α. To compare the effects of treatments and time on mRNA levels, all mRNA measurements were normalized to the quantity of GAPDH mRNA contained in total RNA pools derived from each culture.

Detection of NADPH oxidase components by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from differentiated NHBEC cultures maintained in serum-free NHBEC culture medium supplemented with BEGM by using an RNeasy Qiagen mini kit (Qiagen) and RNase-Free DNase digestion (Qiagen) to eliminate genomic DNA contaminants. Total RNA concentrations were determined by spectrophotometry and adjusted to 50 ng/μl. Messenger RNA contained in total RNA pools derived from each NHBEC donor and human macrophages were reverse transcribed to produce cDNA templates that were amplified by polymerase chain reactions (PCRs) (OneStep RT-PCR Kit; Qiagen) according to the following conditions: 30 min at 50 °C (activation of reverse transcriptase), 15 s at 95 °C (activation of *Taq* polymerase), cycling (40×) for 1 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C. Final polymerase-mediated extension occurred for 10 min at 72 °C. Gene-specific oligonucleotide primers (Table 2) were used in PCRs to amplify cDNAs derived from mRNA species encoding p22*phox*, p41 (*NOXO1*), p47*phox*, p51 (*NOXA1*), p67*phox*, and gp91*phox*. To ensure that the origins of amplification by PCR were derived from cDNA templates, which is indirectly representative of gene expression (mRNA presence), and not from residual genomic DNA contaminants, amplifications by PCRs were conducted in parallel on total RNA pools that did not undergo reverse transcription (reverse transcriptase activation step was eliminated). Equal amounts of total RNA (250 ng) were used for both RT-PCR and PCRs (control).

Amplified products and DNA molecular weight markers (1 kb Plus DNA Ladder; Invitrogen) were separated by electrophoresis in 1% agarose gels. Fragments of interest were extracted using a QIAquick Gel Extraction kit (Qiagen) and the nucleotide sequence of each was determined with use of a Big Dye Terminator (v3.1) Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and model 3970 DNA Analyzer (Applied Biosystems). Nucleotide sequences of each fragment were identified by submission into the nucleotide–nucleotide BLAST program (National Center for Biotechnology Information, USA).

TNF-α ELISA. Tumor necrosis factor-α content in differentiated NHBEC culture-conditioned media was commercially measured (Pierce Biotechnology, Woburn, MA) by ELISA according to the service provider's protocol.

Statistics. Data were analyzed using paired *t* tests and are displayed as means ± standard error of the mean (SEM). A value of $p \leqslant 0.05$ was considered statistically significant.

Results

Cigarette smoke condensate induces H_2O_2 production in NHBECs through activation of NADPH oxidase

To investigate whether CSC induces oxidant production in airway-like NHBEC cultures, we challenged

Table 1
Nucleotide sequences of human probes and primers used for real-time polymerase chain reaction (Taqman) analyses

Gene	Probe (5′–3′)	Primers
MMP-12	CGGGCAACTGGACACATCTACCC	5′-CACTTCTTGGGTCTGAAAGTGA-3′ 3′-GAGGTGCGTGCATCATCTC-5′
Duox1	TCTCCCAGAGTGAGTGCCCGCT	5′-TACATCAGCCAGGATATGATCTGT-3′ 3′-AGGTGTCAACTCAGTCTCAATGTC-5′
TNF-α	TTCTCGAACCCCGAGTGACAAGC	5′-GCCCAGGCAGTCAGATCAT-3′ 3′-TTTGCTACAACATGGGCTACA-5′

Abbreviations: MMP-12, matrix metalloproteinase-12; Duox1, dual oxidase 1; and TNF-α, tumor necrosis factor-α.

Table 2
Nucleotide sequences of human primers used for polymerase chain reaction analyses

Gene	Primers	Predicted fragment size (base pairs)
P22*phox*	5′-GGAGCGCTGGGGACAGAAGTACATG-3′ 3′-GATGGTGCCTCCGATCTGCGGCCG-5′	252
p41 (*NOXO1*)	5′-TGCAGATCAAGAGGCTCCAAAC-3′ 3′-TCTTGAGCTGCCTGAATTCGTC-5′	82
p47*phox*	5′-ACCCAGCCAGCACTATGTGT-3′ 3′-AGTAGCCTGTGACGTCGTCT-5′	768
p51 (*NOXA1*)	5′-AACCATGATGCCAGGTCCCTAA-3′ 3′-AGAGGAGCCTGTTTGCCAACT-5′	139
p67*phox*	5′-CGAGGGAACCAGCTGATAGA-3′ 3′-CATGGGAACACTGAGCTTCA-5′	729
gp91*phox*	5′-TGGTACACACATCATCTCTTTGTG-3′ 3′-AAAGGGCCCATCAACCGCTATCTTAGGTAG-5′	530

Abbreviations: phox, phagocyte oxidase; *NOXO1*, NADPH oxidase organizer 1; and *NOXA1*, NADPH activator 1.

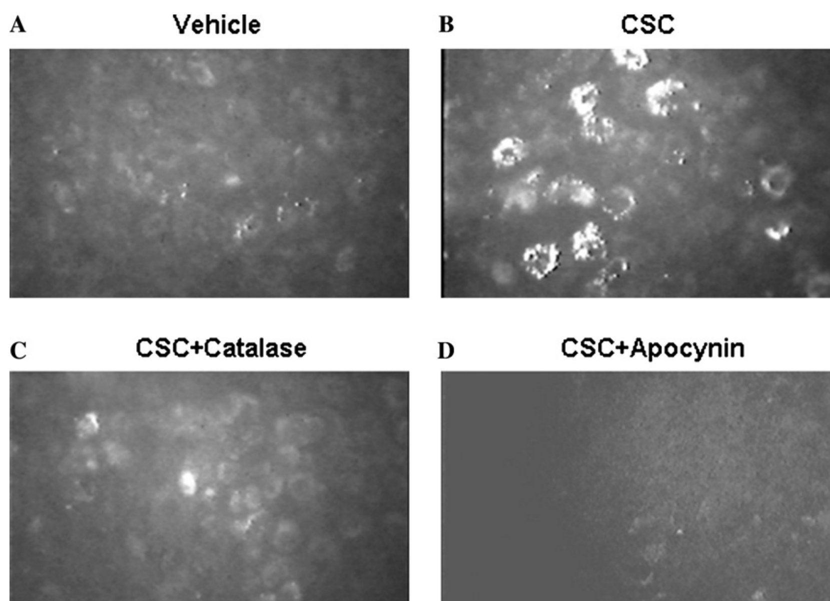

Fig. 1. Cigarette smoke condensate induces H_2O_2 production in airway-like NHBEC cultures through activation of an NADPH oxidase. Dihydrorhodamine-loaded airway-like NHBEC cultures were challenged with vehicle (DMSO; 0.04%), CSC (0.4 μg/ml), CSC plus catalase, or CSC plus apocynin and observed for oxidant production after 60 min. Cultures treated with vehicle exhibited non-specific background fluorescence (A), while CSC-treated cultures displayed robust fluorescent patterns in plasma membranes of cells (B). Absence of fluorescence following co-treatment of cultures with CSC plus catalase (C) or apocynin (D) indicated that CSC stimulated H_2O_2 production through activation of NADPH oxidase. Data are representative of experiments performed on cultures from two independent donors. CSC, cigarette smoke condensate.

DHR-loaded, airway-like NHBEC cultures with CSC (0.4 μg/ml) for 60 min. Although the vehicle (DMSO) failed to induce oxidant generation (Fig. 1A), CSC-induced oxidant production in airway-like NHBEC cultures, as indicated by the robust fluorescence observed in CSC-treated cultures (Fig. 1B). Co-challenge of cultures with CSC and catalase significantly reduced DHR-derived fluorescence emission (Fig. 1C), indicating that CSC-induced H_2O_2 production in airway-like NHBEC cultures. Apocynin, a selective inhibitor of NADPH oxidase [24], blocked CSC-induced oxidant generation in airway-like NHBEC cultures (Fig. 1D). Consistent with this, the membranous fluorescent patterns emitted by CSC-stimulated cells are indicative of involvement of a membrane-bound enzyme, such as an NADPH oxidase.

NADPH oxidase components are expressed in airway-like bronchial epithelial cell cultures

To confirm that NADPH oxidase was expressed in airway-like NHBEC cultures generated in our laboratory, RT-PCR analyses were performed in an attempt to detect mRNA species of classical NADPH oxidase components of phagocytes [11] and of recently described homologues of cytosolic phagocytic components [19–21]. In agreement with previous observations [25], we detected mRNA species corresponding to phagocytic NADPH oxidase components, including p22*phox* (Fig. 2A), p47*phox* (Fig. 2B), p67*phox* (Fig. 2C), and gp91*phox* (Fig. 2D), in airway-like NHBEC cultures. No p41 (*NOXO1*) mRNA was detected (Fig. 2E), but mRNA encoding p51 (*NOXA1*) (Fig. 2F) was found in total RNA pools from airway-like NHBECs.

Consistent with previous findings by provided Geiszt et al. [15], we detected Duox1 mRNA in NHBECs. Fig. 2G shows that CSC (2-h exposure) had no effect on Duox1 mRNA abundance in airway-like NHBEC cultures and indicate that CSC did not regulate *Duox1* expression.

Cigarette smoke condensate increases MMP-12 mRNA abundance in airway-like NHBEC cultures through an H_2O_2-dependent pathway involving NADPH oxidase and AP-1

Considering the significant role played by MMP-12 in CS-induced emphysema [4] and the ability of cultured bronchial epithelia to synthesize MMP-12 mRNA [6], we explored the possibility that CSC could regulate MMP-12 mRNA abundance in airway-like NHBEC cultures.

Fig. 2 demonstrates that, CSC, at a dose (0.4 μg/ml) equivalent to that which induced oxidant production (Fig. 1), regulated MMP-12 mRNA levels in airway-like NHBEC cultures. An approximate 3-fold increase in MMP-12 mRNA levels was apparent within 2 h after CSC stimulation and dissipated with time (Fig. 3A). To examine the roles of oxidants in mediating CSC-induced enhancement in MMP-12 mRNA abundance,

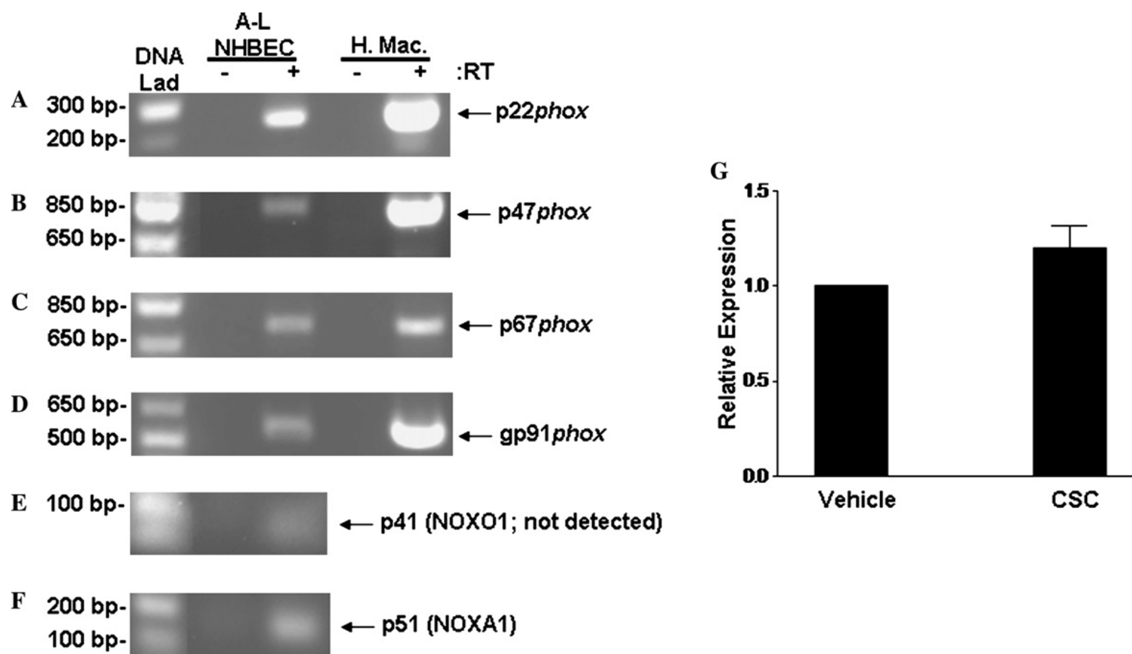

Fig. 2. Genes encoding phagocytic NADPH oxidase components and a homologue of p67*phox* are expressed in airway-like NHBEC cultures. Portions (250 ng) of total RNA pools derived from airway-like NHBEC cultures and human macrophages were subjected to reverse transcription (RT; −) or RT and PCR (+) using gene-specific oligonucleotide primers (Table 1). Messenger RNAs of phagocytic NADPH oxidase components, including p22*phox* (A), p47*phox* (B), p67*phox* (C), and gp91*phox* (D) were detected, albeit in lesser amounts than in macrophages, in airway-like NHBEC cultures. Messenger RNA corresponding to the p47*phox* homologue, p41 (*NOXO1*), was not detected (E), but p51 mRNA (*NOXA1*) (F), the p67*phox* homologue, was found. Real-time PCR analysis shows that gene expression levels of Duox1, a homologue of gp91*phox*, were unmodified by CSC (0.4 μg/ml; 2 h) treatment (G). "Relative expression" describes comparative Duox1 mRNA abundance in vehicle (DMSO; 0.04%)-treated cells and in cultures treated with CSC. Duox1 mRNA levels in vehicle-treated cells were arbitrarily set to "1." These data are representative of experiments performed on total RNA pools of cultures from at least two independent donors. DNA Lad, 1 kb DNA ladder; A-L NHBEC, airway-like normal human bronchial epithelial cell cultures; H. Mac, human macrophages; and CSC, cigarette smoke condensate.

we stimulated airway-like NHBEC cultures with CSC or with CSC in the presence of an oxidant scavenger or an inhibitor of NADPH oxidase for 2 h. Catalase, a scavenger of H_2O_2, significantly reduced the CSC-stimulated increase in MMP-12 mRNA abundance (Fig. 3B). Moreover, apocynin prevented CSC-induced MMP-12 mRNA augmentation (Fig. 3C), which suggested that CSC-induced NADPH oxidase activity was involved in oxidant-dependent MMP-12 expression. Consistent with this, co-application of 3-aminobenzamide, an inhibitor of H_2O_2-sensitive AP-1 [7,26,27], with CSC prevented CSC-induced enhancement in MMP-12 mRNA abundance (Fig. 3D). These findings indicate that an H_2O_2-dependent pathway involving NADPH oxidase and AP-1 mediated CSC-induced elevations in MMP-12 mRNA in airway-like NHBEC cultures.

Cigarette smoke condensate-induced H_2O_2 production and MMP-12 gene expression in airway-like NHBEC cultures involve TNF-α

We considered the possibility that CSC-induced regulation of H_2O_2 production and MMP-12 mRNA abundance in airway-like NHBEC cultures was mediated through autocrine and/or paracrine mechanisms involving a soluble activator or potentiator of NADPH oxidase, such as TNF-α [28]. Tumor necrosis factor-α mRNA levels were greater in airway-like NHBEC cultures treated with CSC for 2 h than in cultures treated with vehicle (Fig. 4A). A similarly modest, yet significant, rise in TNF-α mRNA was previously observed in murine lung following 2 h of CS exposure [29]. The quantity of TNF-α protein in cell culture media conditioned by CSC-treated airway-like NHBEC cultures after 2 h (mean quantity = 31.5 pg/ml ± 4.1 (SEM)) was less, albeit insignificantly ($p = 0.07$), than that detected in media conditioned by vehicle-treated cultures (mean quantity = 47.5 pg/ml ± 9.2 (SEM)). These results agree with those that previously demonstrated induced suppression [30–34] or no effect [35] of CS on TNF-α secretion by macrophages and monocytes. The TNF-α measured in media conditioned by differentiated NHBEC was that which had accumulated during 2 h of culture, since at time zero, all cultures received fresh serum-free, TNF-α-free medium containing a variety of compound additives (as described in Materials and methods; real-time polymerase chain reaction analyses).

To directly investigate a role for endogenously secreted TNF-α in CSC-induced oxidant production and MMP-12 expression, we stimulated airway-like

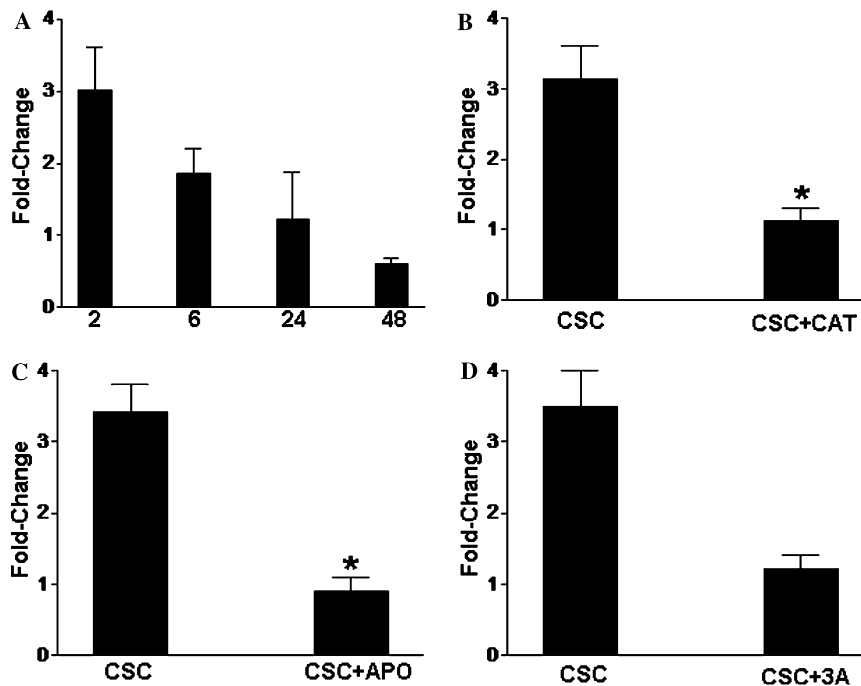

Fig. 3. Cigarette smoke condensate induces MMP-12 expression in airway-like NHBEC cultures through a H_2O_2-dependent pathway involving an NADPH oxidase and AP-1. Airway-like NHBEC cultures were challenged with vehicle (DMSO; 0.04%) or CSC (0.4 μg/ml) for 2, 6, 24, and 48 h. Real-time PCR analyses indicated that CSC-induced MMP-12 expression was greatest 2 h following CSC application to cultures, with a time-dependent decline in expression thereafter (A). "Fold-change" refers to MMP-12 levels detected in CSC-treated cultures relative to levels found in vehicle-treated cultures. Induction of MMP-12 expression in airway-like NHBEC cultures by CSC (2-h exposure) occurred through a H_2O_2-dependent pathway (B) that involved an NADPH oxidase (C) and the H_2O_2-sensitive transcription factor, AP-1 (D). "Fold-change" refers to MMP-12 mRNA levels detected in cultures treated as indicated relative to those in vehicle-treated cultures. A value of "1" indicates no difference. Data are representative of experiments performed on cultures from at least two independent donors. * Significant difference compared to cultures treated with CSC only. In (D), CSC vs. CSC plus 3A is $p = 0.08$. CSC, cigarette smoke condensate; CAT, catalase; APO, apocynin; and 3A, 3-aminobenzamide.

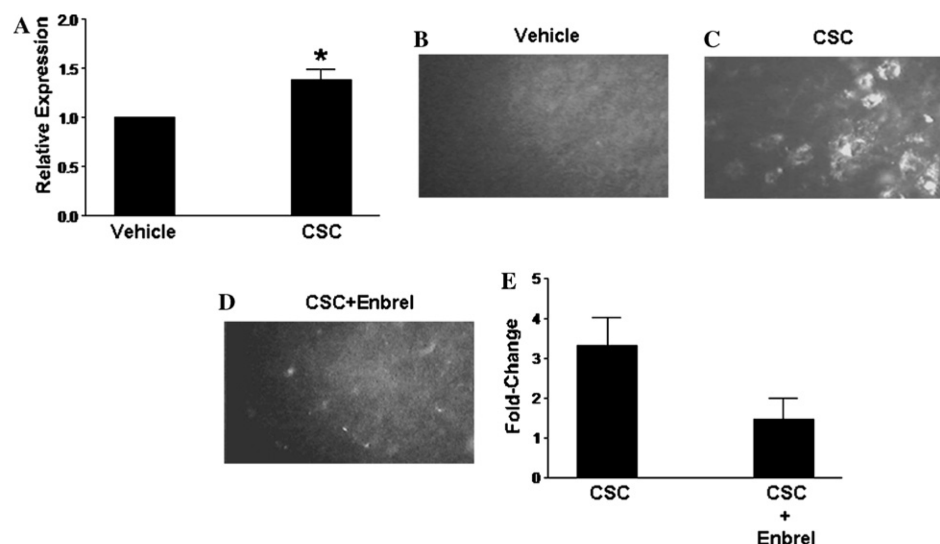

Fig. 4. Cigarette smoke condensate-induced H_2O_2 production and MMP-12 expression in airway-like NHBEC cultures involve TNF-α. Real-time PCR analysis revealed that CSC (0.4 μg/ml; 2-h exposure) increased TNF-α mRNA abundance in NHBEC cultures (A). "Relative Expression" (A) describes comparative MMP-12 mRNA abundance in vehicle (DMSO; 0.04%)-treated cells and in cultures treated with CSC. MMP-12 mRNA levels in vehicle-treated cells were arbitrarily set to "1." Vehicle failed to induce H_2O_2 production (B), while CSC-stimulated (60 min) H_2O_2 generation (C) was inhibited by Enbrel (D), a fusion protein consisting of the ligand binding domain of the 75 kDa TNF-α receptor. In (E), CSC-stimulated (2 h) MMP-12 expression is attenuated by Enbrel. "Fold-change" refers to MMP-12 mRNA levels detected in cultures treated as indicated compared to those in vehicle-treated cultures. A value of "1" indicates no difference. These results demonstrate that TNF-α cooperated with CSC to induce H_2O_2 production and MMP-12 expression in airway-like NHBEC cultures. * Significant difference compared to cultures treated with vehicle. CSC, cigarette smoke condensate.

NHBEC cultures with CSC in the presence of Enbrel, a fusion protein including the ligand binding domain of the 75 kDa TNF-α receptor [36], which binds to TNF-α and, therefore, inhibits TNF-α function. Vehicle administration failed to stimulate H_2O_2 production (Fig. 4B), however, CSC treatment for 60 min promoted H_2O_2 generation in membranous fluorescent patterns (Fig. 4C), as shown previously (Fig. 1B). Enbrel inhibited the ability of CSC to induce H_2O_2 production in airway-like NHBEC cultures (Fig. 4D). Furthermore, co-treatment of airway-like NHBEC cultures with CSC and Enbrel for 2 h attenuated CSC-induced elevations in MMP-12 expression (Fig. 4E). These observations indicated that TNF-α was involved, likely by priming NADPH oxidase activation [28], in CSC-induced H_2O_2 production and MMP-12 expression in airway-like NHBEC cultures.

Discussion

These findings directly show that a concentrated form of the primary cause of emphysema, namely CS, can regulate the expression of MMP-12, which, based on studies in mice [4], is a protein that could play a major role in CS-induced emphysema in humans. Furthermore, we provide evidence that an H_2O_2-dependent pathway mediates the effect of CSC in this in vitro model of airway bronchial epithelium. Cigarette smoke condensate-induced oxidant generation and MMP-12 expression that were inhibitable by catalase or apocynin, an inhibitor of NADPH oxidase [24]. Consistent with this, mRNA species corresponding to phagocytic and novel NADPH oxidase components were detected in total RNA pools derived from airway-like NHBEC cultures. Pharmacological inhibition of AP-1, an H_2O_2-sensitive transcription factor [7] that has a docking site in MMP-12 [8], also reduced CSC-stimulated MMP-12 mRNA levels. Finally, we demonstrate that TNF-α appears to prime CSC-induced H_2O_2 generation and MMP-12 expression in airway-like NHBECs.

Bronchial epithelia are composed of a heterogeneous mix of cell-types, including ciliated columnar epithelial cells, mucus-secreting goblet cells, microvilli-studded brush cells, and basal cells in a pseudostratified arrangement. As such, each cell type performs unique functions that collectively account for the physiological role of this tissue. For example, while epigenetic programs of goblet cells endow them with the ability to secrete mucins/mucous, neighboring ciliated columnar cells express genes such as β-tubulin to construct the cilia required to move mucous to the upper respiratory tract. The mucociliary phenotype of bronchial epithelial cultures used here exhibits a similar cellular heterogeneity [22]. Consequently, the origins of oxidant production and gene expression observed in these cultures may be derived from one or a variety of cell types. Furthermore, the interactions among cell-types may be complex, involving autocrine or paracrine mechanisms to mediate effects of exogenously applied agents such as CS. Indeed, our findings indicated that endogenously secreted TNF-α acted cooperatively with CSC to elicit H_2O_2 production and MMP-12 expression. Consistent with these observations, a role for TNF-α in CS-induced emphysema is associated with positive regulation of MMP-12 [37], elastin breakdown [29,37], and pulmonary matrix degradation [29,37]. Interestingly, the latter phenomenon was not completely inhibited by anti-neutrophil antibodies [37], suggesting that MMP enzyme release from another cell-type, perhaps bronchial epithelial cells, contributed to CS-induced matrix breakdown.

Because H_2O_2 can penetrate lipid, it is conceivable that the cellular origins of MMP-12 expression and the relevant oxidant-producing enzyme, NADPH oxidase, involved in CSC-induced MMP-12 expression may be located in the same or different cell-types. The ability of cell-impermeable catalase to inhibit the CSC-induced increases in MMP-12 mRNA abundance suggests the latter. Our pharmacological and molecular analyses raise the possibility that two forms of NADPH oxidase, which may be expressed in different cell-types within these heterogeneous cell cultures, exist in these airway-like NHBEC cultures and in bronchial epithelia in vivo.

The predominant inducible oxidant-producing enzyme in phagocytes is NADPH oxidase. The phagocytic form of this enzyme can be activated by receptor- or non-receptor-mediated stimuli that initiate intracellular signals to facilitate oxidase assembly and activation [11]. Cigarette smoke may act in one or both of these ways to induce NADPH oxidase activation. Support for direct activation of NADPH oxidase by CS emerged from experiments conducted with cultured endothelial cells [10], where aldehyde components of CS (i.e., acrolein) were presumably responsible for CS-induced oxidant generation that was inhibitable by apocynin or diphenyleneiodonium, a non-specific inhibitor of flavin-containing enzymes such as nitric oxide synthase [38] and NADPH oxidase [39]. NADPH oxidase is selectively inhibited by apocynin [24], which, when activated by a peroxidase, can impede translocation of cytosolic *phox* components to the membrane [40]. In the current study, apocynin attenuated both CSC-induced H_2O_2 production and MMP-12 expression. Apocynin also inhibited proliferation of BECs stimulated by ozone [41] or ultrafine carbon particles [42] and, here, we detected mRNA specific for both p47*phox* and p67*phox* in airway-like NHBECs. Each of these components contributes to oxidant production in non-phagocytic cell-types [43,44], however, their roles in BECs have not been directly shown. Further studies comparing oxidative responses and MMP-12 expression in CSC-stimulated airway-like cultures derived from wild-type and

p47*phox*-deficient mice [45] may resolve this issue. Peroxidase mediated transformation of apocynin into an active metabolite may have occurred through glutathione peroxidase or a newly described alternative form of gp91*phox*, Duox1 [16–18].

Duox1 is a member of a growing list of NADPH oxidase forms that produce oxidants in non-phagocytic cells [14]. Recent work has revealed Duox1 expression in undifferentiated cultured NHBECs, where it exhibited H_2O_2 production [15]. Apocynin-inhibitable p47*phox*/p67*phox* translocation and H_2O_2 production in airway-like NHBEC cultures suggested that Duox1 interacts with these essential phagocytic NADPH oxidase components [46]. However, the expression of gp91*phox* in primary differentiated (airway-like) NHBECs ([25] and herein) raises the possibility that p47*phox*/p67*phox* subunits cooperate with this core NADPH oxidase component to produce H_2O_2 in these cultures. Furthermore, since p51 (*NOXA1*) can interact with p47*phox* to enable superoxide production [19] and contains binding sites for Rac [19–21], it is conceivable that p47*phox* associates with p67*phox* or p51 and Rac to promote Duox1-mediated H_2O_2 generation in airway-like NHBECs (Fig. 5). Clearly, more work is required to directly determine whether p47*phox*, p51, and Rac can interact with each other and, if so, in combination with Duox1 to propagate oxidant production. Future studies aimed at elucidating specific cellular locations of NADPH oxidase components in bronchial epithelia would also assist in resolving this issue.

Regardless of the NADPH oxidase form that indirectly produced H_2O_2 in response to CS, the manifestation of that phenomenon, MMP-12 mRNA accumulation, is remarkable and fits with the proposed role of MMP-12 in CS-induced emphysema in mice [4]. Interestingly, MMP-12 mRNA levels 24 h following the onset of CSC exposure were nearly back to baseline levels, suggesting that oxidant-dependent mechanisms driving MMP-12 expression had ceased by this time. These observations agree with those made previously in lung epithelial monolayers, which described an initial drop in anti-oxidant glutathione levels with CSC

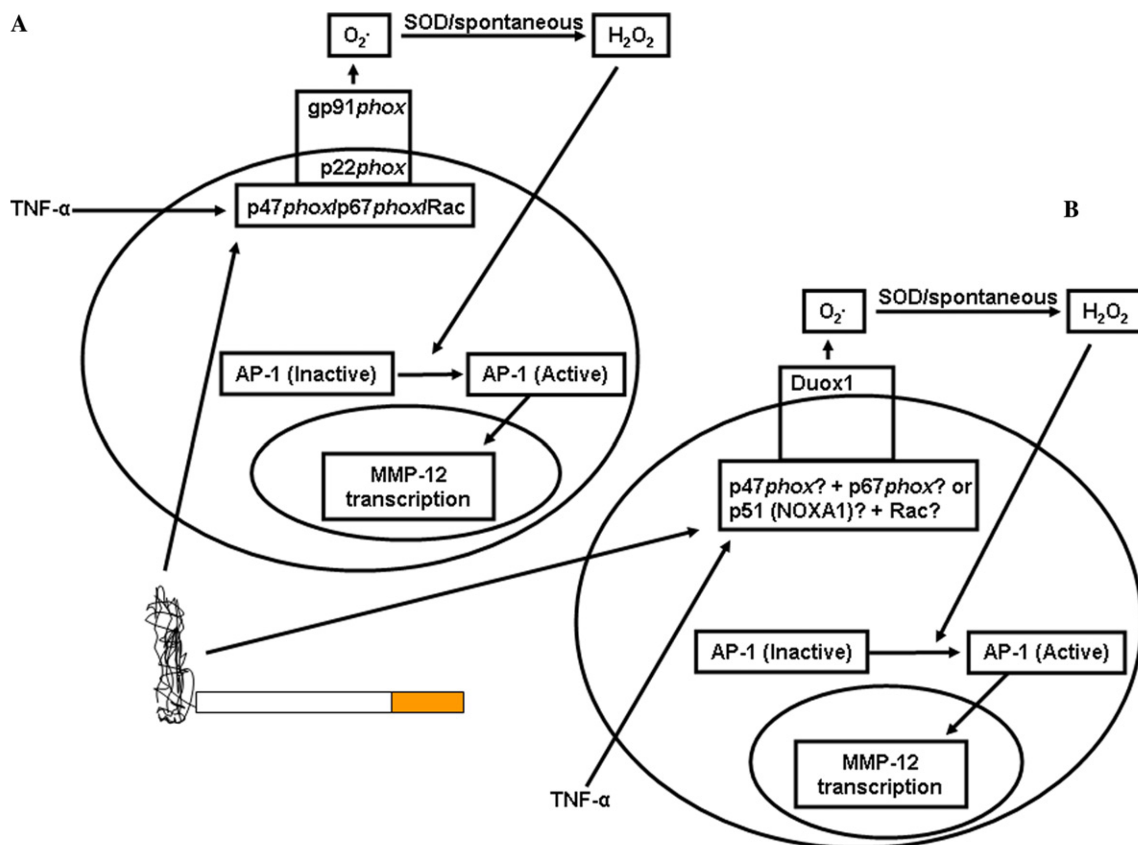

Fig. 5. Proposed models of CSC-induced oxidant production and MMP-12 expression in airway-like NHBEC cultures. Two CSC-inducible forms of NADPH oxidase may exist in bronchial epithelia, based on findings in heterogeneous cellular airway-like NHBEC cultures. In (A), phagocytic components comprise NADPH oxidase and are cooperatively activated by CS and TNF-α to primarily produce superoxide anions ($O_2^{\cdot}$) that can undergo dismutation by SOD or spontaneously to form H_2O_2. Hydrogen peroxide-activated AP-1 can contribute to initiation of MMP-12 transcription. In (B), Duox1 is putatively expressed in a different cell-type of the bronchial epithelium where it may interact with cytosolic oxidase components, including p47*phox* plus p67*phox* or p51, and Rac.

exposure followed by restoration of glutathione abundance 24 h after CSC challenge [47]. Recovery of glutathione levels likely involved CSC-induced activation of AP-1 [48,49], which can mediate CS-regulated gene expression of the rate limiting enzyme of glutathione synthesis, γ-glutamylcysteine synthetase [49]. In our study, then, it is possible that AP-1 function was dichotomously manifested, both as facilitator and, by putatively enhancing glutathione levels, as terminator of MMP-12 expression.

In summary, we have provided novel evidence for a direct relationship between CS and MMP-12 in human cells. Additionally, these data suggest a mechanism through which CS can control MMP-12 expression in bronchial epithelia in vivo. These findings apply biological relevance, albeit pathological, to MMP-12 expression in bronchial epithelia [6]. The reactivity of these cells, by virtue of their potential to release MMP-12 in vivo, may be critical for determining the manifestations of CS exposure in lung. Considering the results presented here and their implications, further studies in MMP-12- and NADPH oxidase component-deficient "knock-out" mice are warranted to investigate the notion that CS can induce MMP-12 expression through NADPH oxidase-mediated, oxidant-dependent pathways in bronchial epithelia in vivo. Such experiments may support development of pharmacological strategies to oppose CS-induced emphysema in humans.

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